IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re PATENT APPLICATION of:

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Filed: April 6, 2000 EXAMINER: Gollamudi S. Kishore, Ph. D

For: LIPID METABOLISM IMPROVING AGENT

DECLARATION PURSUANT TO 37 C.F.R. 1.132

Sir:

I, Satoshi Nagaoka, of Gifu-Shi, Gifu 501-1193, Japan hereby declare as follows,

I graduated from Department of Agricultural Chemistry, Faculty of Agriculture, Utsunomiya University in March, 1981,and graduated from Department of Agricultural Chemistry, Faculty of Agriculture, Nagoya University in March, 1986, and got a doctor's degree in nutritional biochemistry for a thesis entitled "Comparative studies on the hypercholesterolemia induced by dietary xenobiotics or dietary excess tyrosine in rats" from Nagoya University in April, 1987.

Research Experience:

1988-1989 Nagoya University, Japan, Awardee of the Fellowships of Japan Society for the Promotion of Science for Japanese Junior Scientists, Nutritional

Biochemistry

1989-1993 Gifu University, Research Assistant, Nutritional Biochemistry

1994-1995 Boston University, School of Medicine, USA, Overseas Research Scholar (Ministry of Education), Biochemistry, Molecular Genetics

1993- Gifu University, Faculty of Agriculture, Japan, Associate Professor Nutritional Biochemistry

1996 Awardee of the Encouragement Award of the Japanese Society of Nutrition and Food Science

2005 Awardee of the Commendation for Science and Technology by the Minister of Education, Culture, Sports, Science and Technology, Prizes for Science and Technology (Research Category)

I am one of the co-inventors of the invention described and claimed in the application and have full knowledge of the present invention and cited references.

I conducted the following experiment to examine the effect of protein hydrolyzate/enzyme-modified phospholipid complex in improving lipid metabolism.

[Materials and methods]

Preparation of soy protein hydrolyzate

Soy protein (New Fujipro-E; Fuji Oil, Osaka, Japan) was hydrolyzated by

pepsin (activity; 1:10,000, Nacarai Tesque, Kyoto, Japan) at pH2, and the solution was incubated at 37°C for 24 hours. Pepsin (1g/100g) was added to the protein. The reaction was stopped by heating at 90°C for 30minutes, and the mixture was neutralized with 2M NaOH. After diluting the solution with three volumes of water, the solution was centrifuged at 4,500 × g for 10minutes and the precipitate was recovered. The precipitate was freeze-dried to obtain soy protein hydrolyzate.

Preparation of soy protein hydrolyzate /enzyme-modified phospholipid complex

Soy protein (New Fujipro-E; Fuji Oil, Osaka, Japan) was dispersed in water and then stirred at 10,000 rpm for 5 minutes to make a solution. Enzyme-modified phospholipid (Elmizer AC; T&K lecithin, Mie, Japan) which was prepared by hydrolyzing soy phospholipid with phospholipase A_2 was then added to the solution such that the ratio of soy protein and enzyme-modified phospholipid was 4:1. The mixture was stirred at 10,000 rpm for 5 minutes to prepare a solution containing soy protein/ enzyme-modified phospholipid complex. Pepsin (activity; 1:10,000, Nacarai Tesque, Kyoto, Japan) was then added at 1% (w/w) at pH2, and the solution was incubated at 37°C for 24 hours. The reaction was stopped by heating at 90°C for 30minutes, and the mixture was neutralized with 2M NaOH. After diluting the solution with three volumes of water, the solution was centrifuged at 4,500 × g for 10minutes and the precipitate was recovered. The precipitate was freeze-dried to obtain soy protein hydrolyzate /enzyme-modified phospholipid complex.

Animals and diets

Male rats of the Wistar strain (Japan SLC, Hamamatsu, Japan) were used. Room temperature was maintained at 22 ± 2℃ with a 12 hour cycle of light (8:00-20:00) and darkness. All of the rats were housed individually in metal cages and were allowed free access to diets and water. After acclimation to a commercial nonpurified MF diet (Orient yeast, Osaka, Japan) for 3days, rats were divided into three groups on the basis of body weight. The composition of the basal diet, as recommended by the American Institute of Nutrition (1977), was shown in Table 1. Soyprotein(New Fujipro-E; Fuji Oil, Osaka, Japan), Soy protein hydrolyzate and Soy protein hydrolyzate / enzyme-modified phospholipid complex, which were prepared above, were added to the diet at the nitrogen level equivalent to that of Soy protein-diet at the expense of carbohydrate. Methionine, an essential amino acid, was added into the basal diet in order to make the nutritive values of both compositions equal.

Table 1. Composition of the diets (g/kg)

	Soyprotein	Soyprotein hydrolyzate	Soyprotein hydrolyzate/ enzyme-modified phospholipid complex
Soyprotein	231.75		
Soyprotein hydrolyzate		244.20	
Soyprotein hydrolyzate / enzyme-modified complex			323.10
Lard	50	50	50
Corn oil	10	10	10
Mineral mixture*	35	35	35
Vitamin mixture*	10	10	10
Choline chloride	2	2	2
Sucrose	200.99	202.15	170.77
Celllose	50	50	50
Cholesterol	5	5	5
Sodium cholate	2.5	2.5	2.5
Corn Starch	401.98	389.15	341.53
Methionine	0.78	0	0.1

* AIN-76 diet (American Institute of Nutrition, 1977).

Rats were divided into 3 groups of 6 rats on the basis of body weight. Each group was fed freely one of the respective test diets containing soy protein, soy protein hydrolyzate and soy protein hydrolyzate /enzyme-modified phospholipid complex as the protein source for 10days. Each rat was measured its body weight everyday throughout this experiment with an electronic balance. After fasting for 24 hours, the rats were anesthetized with diethyl ether. Blood was collected by cardiac puncture and the liver removed. Each liver was weighed with an electronic balance after rinsed with ice-cold saline.

Lipid analysis

Various lipid concentrations were determined using commercially available kits as follows: serum and liver cholesterol with Monotest cholesterol (Boehringer Mannheim Yamanouchi, Tokyo, Japan); HDL cholesterol with HDL-cholesterase (Nissui, Tokyo, Japan); serum and liver triacylglycerol with Triglycolor III (Boehringer Mannheim Yamanouchi, Tokyo, Japan); serum phospholipids with Phospholipid C-Test Wako (Wako Pure Chemical, Osaka, Japan). Liver lipids were extracted with chloroform-methanol (2:1, v/v) in accordance with Folch partition method, and total lipids were determined gravimetrically.

Statistical analysis

Results are expressed as means and pooled SEM. The statistical significance of differences was evaluated by Duncan's multiple-range test after one-way ANOVA. The significance levels quoted are two-sided. Results were

considered significant at $P<0.05$.

[Results]

The results are shown in Table 2.

Table 2. Body weight gain, food intake, liver weight, serum and liver lipids in rats (Mean values with standard errors of mean for six rats†)

	Diet group			
	Soyprotein	Soyprotein hydrolyzate	Soyprotein hydrolyzate/ enzyme-modified phospholipid complex	Pooled SEM
Body weight gain,g/10d	27.4	29.9	25.3	1.8
Food intake,g/day	15.1	15.7	14.5	0.4
Liver weight,g/100g body weight	3.80 ^{a}	3.83 ^{a}	3.52 ^{b}	0.08
Serum, mmol/L				
Total Cholesterol (a)	2.69	2.21	1.95	0.32
HDL cholesterol (b)	0.90 ^{c}	1.18 ^{b}	1.38 ^{a}	0.06
LDL+VLDL cholesterol ††	1.79 ^{a}	1.03 ^{ab}	0.57 ^{b}	0.31
(a)/(b), mol/mol	0.33 ^{c}	0.54 ^{b}	0.71 ^{a}	0.03
Triacylglycerol	0.66	0.70	0.67	0.10
Phospholipids	1.36	1.39	1.38	0.08
Liver,				
Total lipids,mg/g liver	127.2 ^{a}	88.2 ^{b}	59.9 ^{c}	3.2
Cholesterol, μmol/g liver	74.3 ^{a}	37.2 ^{b}	11.1 ^{c}	2.8
Triacylglycerol, μmol/g liver	30.1 ^{a}	21.8 ^{b}	10.2 ^{c}	1.9
Phospholipids, μmol/g liver	93.2 ^{a}	71.5 ^{b}	60.2 ^{c}	2.1

* For details of diets, see Table 1.

† Values are means (n=6) and pooled SEM.
Within a row, means with different superscript letters are significantly different ($p<0.05$) by Duncan's multiple range test.

†† Values were calculated as follows: LDL+VLDL cholesterol = total cholesterol - HDL cholesterol.

[Conclusion]

As shown in Table 2, there were no significant differences in body weight gain and food intake among the group. The content of serum total cholesterol of the soy protein hydrolyzate / enzyme-modified phospholipid complex fed group was lower than that of soy protein hydrolyzate fed group. And the contents of serum LDL+VLDL

cholesterol, liver total lipids, liver cholesterol, liver triacylglycerol and liver phospholipids of the soy protein hydrolyzate / enzyme-modified phospholipid complex fed group were significantly lower than those of the soy protein hydrolyzate fed group. Further, the content of serum HDL cholesterol and the ratio of HDL cholesterol to total cholesterol of soy protein hydrolyzate / enzyme-modified phospholipid complex fed group were significantly higher than those of the soy protein hydrolyzate fed group.

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: This 23th day of June , 2005.

Satoshi Nagaoka

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